Ets-2 in Pancreatic Cancer Associated Fibroblasts Promotes Tumor Initiation and Development.

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Abstract

Pancreatic cancer remains an overwhelmingly fatal disease with less than 5% of patients surviving beyond 5 years, largely due to our lack of understanding of the complexity of the disease. Many recent reports have begun to highlight the potential role that stromal cells—fibroblasts in particular—may have on pancreatic tumor cell biology and this report provides data that supports the theory of tumor-stroma co-evolution in pancreatic cancer. Here we use a novel mouse model to show that Ets-2 in the tumor-associated stroma promotes pancreatic tumor initiation and development. We observed a decrease in tumorigenesis events such as acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) lesions when Ets-2 is conditionally deleted in pancreatic cancer associated fibroblasts (CAFs). To determine how Ets-2 in fibroblasts is able to effect tumor progression, we harvested pancreatic CAFs from Ets-2 deleted and Ets-2 intact tumor bearing mice and performed microarray gene expression analysis. We found that Ets-2 deleted CAFs had a significantly altered secretome that crucially lacked tumor necrosis factor alpha (TNF-α), a known pro-tumor ligand in pancreatic carcinogenesis. Furthermore, ChIP analysis showed that Ets-2 binds the proximal promoter of TNF-α to directly regulate its expression. Thus, Ets-2 ablation in pancreatic fibroblasts delays pancreatic tumor initiation by decreasing the pro-tumor ligand TNF-α. This report shows for the first time that deleting a gene in pancreatic fibroblasts causes a change in tumor-stroma co-evolution and that

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Ets-2 is able to act as a novel oncogene in cancer associated fibroblasts to promote pancreatic carcinogenesis.

Introduction

Pancreatic cancer is an overwhelmingly fatal disease with approximately 95% of patients dying within 5 years of diagnosis [Siegel et al., 2013]. Moreover, our lack of knowledge in pancreatic cancer is evident in the fact that it has the least significant improvement in overall survival rate amongst all cancers over the last 35 years [Siegel et al., 2013]. Early studies on this disease focused on uncovering the genetics behind tumor formation, which led to the discovery of KRAS, CDKN4A, TP53, and SMAD4 mutations within tumor cells [Almoguera et al., 1998, Caldas et al., 1994, Redston et al., 1994, Hahn et al., 1996]. These studies were all performed in order to better understand the genetics behind Pancreatic Ductal Adenocarcinoma (PDAC), the most clinically prevalent form of pancreatic cancer. Recently, researchers have begun to study earlier events in pancreatic tumorigenesis and it has become apparent that PDAC originates from premalignant lesions termed Pancreatic Intraepithelial Neoplasms (PanIN) that arise from activating KRAS mutations in the epithelium [Hezel et al., 2006]. These efforts have also uncovered potential tumor initiating transformation events where pancreatic acinar epithelial cells trans-differentiate into ductal cells (termed acinar-to-ductal metaplasia, ADM) and then progress to PanIN [De La O et al., 2008]. Importantly, this transition from ADM to PanIN to PDAC is associated with a dramatic expansion of the pancreatic stroma, particularly in the fibroblast compartment. Thus far, the genetics of the tumor microenvironment during pancreatic tumor formation have been largely ignored. This report looks to further the understanding of the role that the pancreatic stroma plays in epithelial transformation using genetically engineered mouse models.

Ets-2 (v-ets erythroblastosis virus E26 onocogene homologue 2) is a proto-oncogene in the Ets family of transcription factors that is overexpressed in a variety of cancers, including PDAC [Ito et al., 2002]. Furthermore, Ets-2 has previously been shown to play a driver role in CAFs in mammary tumorigenesis [Trimboli et al., 2009]. However, it has yet to be determined the role that stromal Ets-2 plays in pancreatic tumor development. In this study, we describe the generation of a genetically engineered mouse model of fibroblast-specific knockout of Ets-2 in Kras-driven pancreatic tumors. Notably, we identify an oncogenic role for Ets-2 in pancreatic CAFs and show that loss of Ets-2 in fibroblasts leads to decreased PanIN and ADM lesions. Gene expression analysis of Ets-2 deleted pancreatic CAFs indicates that the secretome is misregulated upon loss of Ets-2, specifically showing a decrease in the pro-tumor ligand TNF-α. We also show that Ets-2 directly regulates TNF-α production at the transcriptional level in pancreatic CAFS and loss of Ets-2 leads to decreased *Tnfa* expression. Collectively, this report shows that pancreatic CAFs are dependent on Ets-2 in order to create a TNF-α induced pro-tumor microenvironment.

Materials and Methods

Mice

The Mist1-Kras^{G12D}, Ets-2^{LoxP} (herein referred to as Ets-2^{fl}), Ets-2^{db}, and Fsp-Cre alleles have all been previously described [Tuveson et al., 2006, Wei et al., 2009, Yamamoto et al., 1998, and Trimboli et al., 2008]. The use of animals was in compliance with federal and University Laboratory Animal Resources (ULAR) at The Ohio State University regulations and was conducted under the protocol (2007A0120-R1) which was reviewed and approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC).

Tumor Studies

Mist1-Kras^{G12D} tumor bearing mice were sacrificed from 15-30 weeks after birth as indicated. At the time of euthanasia, total body weight and pancreas/tumor weight were documented. Pancreata, liver, and lung were removed and either frozen in OCT or fixed in formalin for 48-72 hours for subsequent histological processing by the Soild Tumor Pathology Core at OSU.

Pancreatic Cancer Associated Fibroblast Isolation

Mist1-Kras^{G12D} mice were euthanized and individual pancreata were removed and minced in 5mg/ml Type II collagenase in PBS. After mincing, pancreata were dissociated by shaking at 225 RPM at 37°C for 60 minutes. Cells were washed with complete Dulbecco's Modified Eagle Medium (DMEM, containing 10% FBS) and then pelleted by centrifugation at 200xg for 5 minutes at 4°C. Cell pellets were resuspended in 5ml complete DMEM and allowed to gravity precipitate at room temperature for 10 minutes. After precipitation, top 3ml of solution was discarded and cellular pellet was resuspended in up to 5ml of complete DMEM, thus constituting one round of gravity precipitation. An additional round of gravity precipitation was conducted and the final cellular pellet was seeded. 24-hours post seeding media was replaced with complete DMEM. To further purify fibroblasts cultures, selective trypsonization was utilized where 0.25% trypsin was added to PBS-washed plated for 90 seconds and then fibroblasts were collected and re-seeded on a new plate. Fibroblast purity was confirmed by immunofluorescence as noted.

Immunofluorescence and Histological Staining of Tissue Sections

After removal from the body, tissue was immediately frozen in OCT or fixed in formalin for 48 hours, transferred to 70% ethanol and then paraffin embedded. Frozen or embedded tissue was subsequently sectioned and mounted on glass slides for further analysis. Sections were processed

with xylenes and ethanol prior to DAKO antigen retrieval in a streamer for 30 minutes. Sections were blocked with DAKO protein block and tissues were stained with anti- F4/80 (Invitrogen), -vimentin (Cell Signaling), beta amylase (Cell Signaling), -cytokeratin 19 (Iowa Developmental Studies Hybridoma Bank), and -Ki67 (Abcam) as noted. Hematoxylin and eosin (H&E) and Masson's Trichrome staining was performed by the Solid Tumor Pathology Core at OSU.

Immunofluorescence Staining of Fibroblast Cultures

Cells were grown on glass cover slips to 90% confluence and fixed using 4% PFA. Fixed cells were blocked using DAKO protein block and stained with anti-α smooth muscle actin (Abcam) and –cytokeratin 19 (Iowa Developmental Studies Hybridoma Bank) as noted.

RNA Isolation and Quantitative Real Time PCR

RNA was isolated using Trizol reagent (Invitrogen), treated with DNase I (Ambion), and cDNA was generated using Superscript III Reverse Transcription (Invitrogen) as per the manufacturer's specifications. Quantitative real time PCR was performed using primer specific Roche Universal Probe Library system on Applied Biosystems Step One Plus real time PCR systems.

Gene Set Enrichment Analysis (GSEA)

GSEA v2.0 was downloaded from the BROAD institute (http://www.broadinstitute.org/gsea) and used to determine molecular pathways that were misregulated upon deletion of Ets-2. Gene sets were obtained from Broad institute GSEA and ToppGene Suite databases. Statistical analysis was determined using 1,000 random permutations of each gene set to obtain a nominal P value and normalized enrichment score (NES).

Results

Mist1-Kras^{G12D} Mice Developed Dramatic Stromal Reaction

Mist-Kras^{G12D} mice have been previously shown to develop mixed differentiated pancreatic carcinoma with the accompaniment of a rich stromal component, similar to human tumors [Tuveson et al., 2006]. We further characterized the Mist1-Kras^{G12D} mouse tumor model by performing a time-course analysis of the stromal reaction as the disease progresses. We monitored tumor histology on a bi-weekly basis and determined that 23 weeks of age consistently lead to increased collagen deposition in Mist1-Kras^{G12D} mice pancreata relative to wild type mice, as measured by Masson's Trichrome staining (Figure 1A-B). Histologically, 23-week old Mist1-Kras^{G12D} mice have developed PanIN and ADM lesions, but are still premalignant. Furthermore, we showed a dramatic increase in SMA+ fibroblasts and F4/80+ macrophages in 23-week old Mist1-Kras^{G12D} pancreata (Figure 1C-D). These data indicate that the Mist1-KrasG12D mouse pancreatic tumor model is a valid system for us to study the role of pancreatic CAFs in tumor initiation and development.

Ets-2 is Efficiently Deleted in Pancreatic Cancer Associated Fibroblasts

In order to conditionally delete Ets-2 in pancreatic fibroblasts in the Mist-Kras^{G12D} pancreatic tumor model, we generated Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$ experimental mice and Mist1-Kras^{G12D}; Ets-2$^{db/fl}$ control mice. We utilized the previously validated Ets-2^{db} null allele that lacks the DNA binding domain and is functionally null in order to increase the efficacy of our cre-loxP deletion of the Ets-2^{fl} allele [Wallace et al., 2013, Trimboli et al., 2009, Zabuawala et al., 2010]. Using a novel procedure for harvesting pancreatic fibroblasts (Figure 2A), we were able to isolate over 99% pure populations of fibroblasts as measured by the expression of fibroblast marker SMA and lack of expression of epithelial marker cytokeratin 19 (Figure 2B).

Furthermore, we showed that these cells also expressed alternative fibroblast marker vimentin and lacked expression of cytokeratin 8 (data not shown). After verifying that novel method of purifying fibroblasts was effective, we performed conventional genotyping PCR on genomic DNA isolated from wild type, Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$, and Mist1-Kras^{G12D}; Ets-2$^{db/fl}$ fibroblast cultures. We showed that Ets-2 was deleted exclusively in pancreatic fibroblasts from Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$ mice using loxP-recombination specific primers (Figure 2C, left). In order to show that the Ets-2^{fl} was also excised *in vivo*, we isolated genomic DNA from formalin fixed paraffin embedded (FFPE) pancreatic tissue sections from Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$, and Mist1-Kras^{G12D}; Ets-2$^{db/fl}$ mice and performed genotyping using the same recombination specific PCR primers (Figure 2C, right). We saw cre deletion specific amplicons exclusively in Mist1-Kras; Fsp-Cre; Ets2$^{db/fl}$ animals. To confirm that genomic Ets-2 deletion leads to decreased mRNA production, we performed quantitative real time PCR on RNA isolated from Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$, and Mist1-Kras^{G12D}; Ets-2$^{db/fl}$ fibroblasts (Figure 2D). Importantly, our cre-deleted mice have one remaining Ets-2^{db} allele that will make mRNA, but not functional protein [Yamamoto et al., 1998]. Therefore, we expect to see ~50% decrease in cre-deleted Ets-2$^{db/fl}$ mice, indicating high recombination efficiency of the Ets-2^{fl} allele. Collectively, this data shows that we can generate pure populations of murine pancreatic CAFs and that Ets-2^{fl} alleles are excised in fibroblasts in Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$ mice.

Loss of Ets-2 in Pancreatic Cancer Associated Fibroblasts Decreases Premalignant Lesions in the Mist1-Kras^{G12D} Murine Pancreatic Tumor Model.

In order to show the effect of Ets-2 loss in pancreatic fibroblasts on tumorigenesis events, we monitored pancreatic tumor development by H&E staining from Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$, and Mist1-Kras^{G12D}; Ets-2$^{db/fl}$ mice at 23 weeks of age. Histological analysis showed that

mice lacking Ets-2 in pancreatic CAFs have delayed onset of Pancreatic Intraepithelial Neoplastic (PanIN) lesions relative to Ets-2 intact age-matched controls (Figure 1A). Additionally, we showed that the same mice have significantly decreased acinar-to-ductal metaplastic (ADM) lesions as measured by ducts that are double positive for epithelial cell marker cytokeratin 19 (K19) as well as acinar cell marker amylase (Figure 1B). In order to explain the decrease in premalignant lesions, we stained pancreatic sections for the ductal cell marker cytokeratin 19 and the cellular proliferation marker Ki67 and showed that Ets-2 fibroblast deleted mice had decreased percentage of proliferating ductal tumor cells. Thus, these results indicate that fibroblast specific deletion of Ets-2 in the pancreas leads to a significant delay in premalignant PanIN and ADM lesion formation due to a decrease in the proliferation potential of the pancreatic epithelium.

Ets-2 Regulates a Pro-Tumor Secretome in Pancreatic Cancer Associated Fibroblasts

To understand how deleting Ets-2 may change the proliferation potential of the adjacent tumor cell, we isolated CAFs from Mist1-Kras^{G12D}; Fsp-Cre; Ets-2$^{db/fl}$, and Mist1-Kras^{G12D}; Ets-2$^{db/fl}$ pancreata and performed microarray (Figure 4A). Gene expression analysis followed by Gene Set Enrichment Analysis (GSEA) determined that Ets-2 deletion significantly altered the secretory pathway within fibroblasts (Figure 4B). We generated a heat map to depict significant changes in select genes whose secreted protein products may act upon the adjacent epithelia (Figure 4C). Not surprisingly, many pro-tumor secreted factors as well as matrix remodelers such as the MMP, CXCL, and CCL were decreased in Ets-2 deleted fibroblasts. This result mimicked previously described results for Ets-2 deletion in mammary tumor models. Interestingly a novel fibroblast Ets-2 regulated factor, tumor necrosis factor alpha (TNF-α), appeared in this analysis to be downregulated at the mRNA level in our microarray upon Ets-2

deletion (Figure 4C). Additionally, GSEA showed that TNF-α pathways was significantly altered upon Ets-2 deletion, further confirming that Ets-2 regulates TNF-α. This result was significant given the previously published importance of TNF-α in many pro-tumor processes in pancreatic and other cancers [Balkwill et al., 2006, Liou et al., 2013].

Ets-2 Drives TNF-α Production in Pancreatic Fibroblasts

To confirm the microarray result that Ets-2 regulates TNF-α expression, we performed quantitative real time PCR and showed that *Tnfa* mRNA was significantly downregualted in Ets-2 deleted fibroblasts (Figure 5B). This result indicates that Ets-2 regulates *Tnfa* expression, but does not prove that Ets-2 directly binds to and induces transcription of *Tnfa*. However, Ets-2 has been speculated to bind to the proximal promoter of *Tnfa* due to the consensus binding sequence -115 basepairs upstream of the transcription start site (TSS), but no lab has directly shown Ets-2 binding to our knowledge. To demonstrate that Ets-2 directly regulates *Tnfa* expression by binding to its proximal promoter, we performed Chromatin Immunoprecipitation (ChIP) for Ets-2 and IgG control and performed quantitative real time PCR for *Tnfa*. We showed significant enrichment for Ets-2 at the *Tnfa* promoter relative to IgG pulldown in Ets-2 intact CAFs. Moreover, we confirmed lack of Ets-2 pulldown in Ets-2 knockout CAF lines (Figure 5C). Together this data suggests that Ets-2 regulates *Tnfa* expression by directly binding to its promoter and inducing transcription.

Discussion

Ets-2 has been documented to play an important role in the effect of the stroma on epithelial transformation and tumorigenesis of cancers of other organ systems [Wallace et al., 2013, Bronisz et al., 2011, and Trimboli et al., 2009]. These results are not surprising given the known

Ets-2-mediated pathways such as extracellular matrix (ECM) remodeling, angiogenesis, cellular proliferation, and cell migration [Wallace et al., 2013]. Additionally, these pathways have been speculated to play a role in the development of pancreatic tumors and we hypothesized that disrupting Ets-2 signaling may influence tumor development.

In this study, we demonstrate that Ets-2 is a key component in the promotion of pancreatic tumor formation by fibroblasts, by showing significantly delayed tumorigenesis events in fibroblast Ets-2 conditional fibroblast deletion mice. This finding is significant because it is the first time that conditional deletion of a gene in fibroblasts has been shown to affect pancreatic tumor growth and development. Additionally, we have provided mechanistic insight into how Ets-2 in fibroblasts is able to influence epithelial cell malignancy by showing that Ets-2 directly binds to the *Tnfa* promoter. Thus, deletion of Ets-2 in fibroblasts leads to decreased TNF-α production, which is a known pancreatic tumorigenesis modulator [Liou et al., 2013]. Currently ongoing studies are required to show that fibroblast-derived TNF-α is necessary and/or sufficient to induce ADM and PanIN both *in vitro* and *in vivo*. Additionally, we will further elucidate the mechanistic details of the Ets-2 regulated pathways through co-culture experiments. In brief, we will co-culture pancreatic epithelial cells and pancreatic fibroblasts (either intact or deleted for Ets-2) and measure the effect of deleting Ets-2 upon epithelial cell proliferation and *in vitro* ADM formation, as previously described (in vitro ADM paper). Additionally, we will harvest conditioned media from Ets-2 intact or deleted fibroblasts and treat normal pancreatic epithelial cells to see if direct cell-to-cell contact is required for the transformative abilities of fibroblasts. Together, these experiments will develop a more complete story of the role of pancreatic fibroblast Ets-2 and provide mechanistic insight into the pathways that Ets-2 regulates in pancreatic CAFs.

In conclusion, this study has established an oncogenic role for Ets-2 in pancreatic fibroblasts through a TNF-α mediated mechanism. This result shows for the first time that deleting a gene in pancreatic fibroblasts is able to influence the fate of the tumor cell and supports the hypothesis that pancreatic cancer associated fibroblasts play a driver role in determining the fate of the tumor cell.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

Acknowledgments

Figure 4A image of GeneChip® Scanner 3000 7G adapted from Affymetrix support website. We graciously thank the Solid Tumor Pathology Core at OSU for their technical assistance in processing tumor samples.

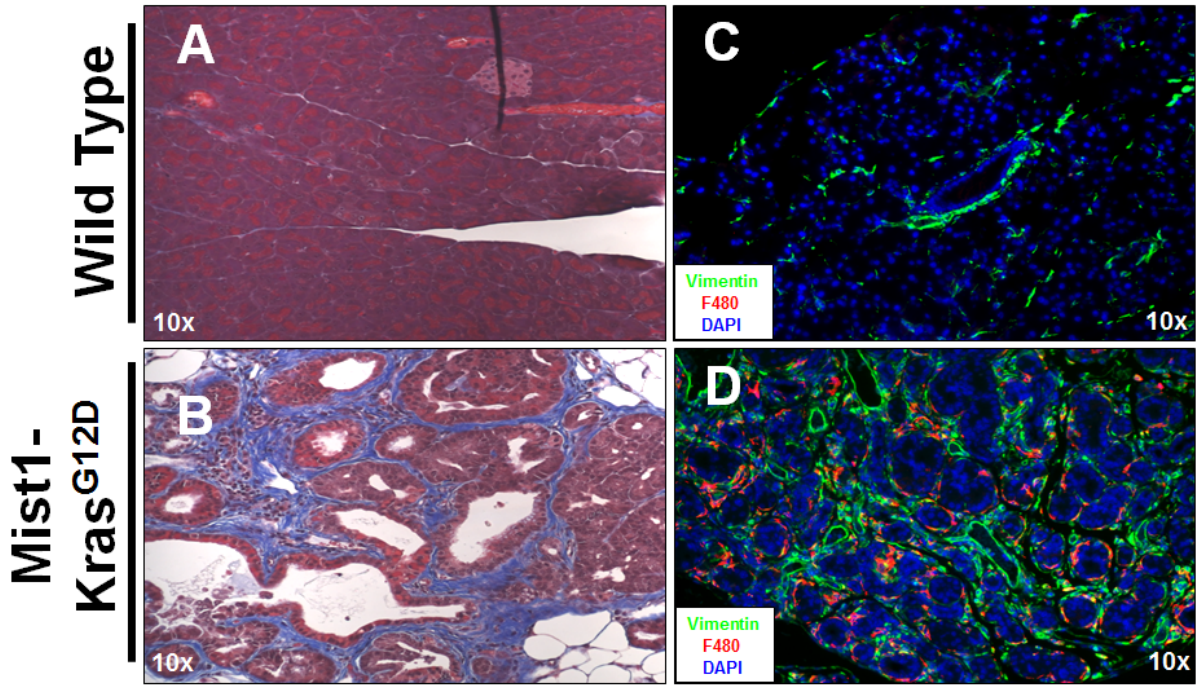

Figure 1. Mist1-Kras^{G12D} Mice Develop Dramatic Stromal Reaction.
(A,B) Trichrome staining showing Mist1-Kras^{G12D} mice develop PanIN and ADM lesions with significant stromal invasion at 23 weeks; histology resembles human condition.
(C,D) Mist1-Kras^{G12D} mice pancreata show increased vimentin+ fibroblast and F4/80+ macrophage populations.

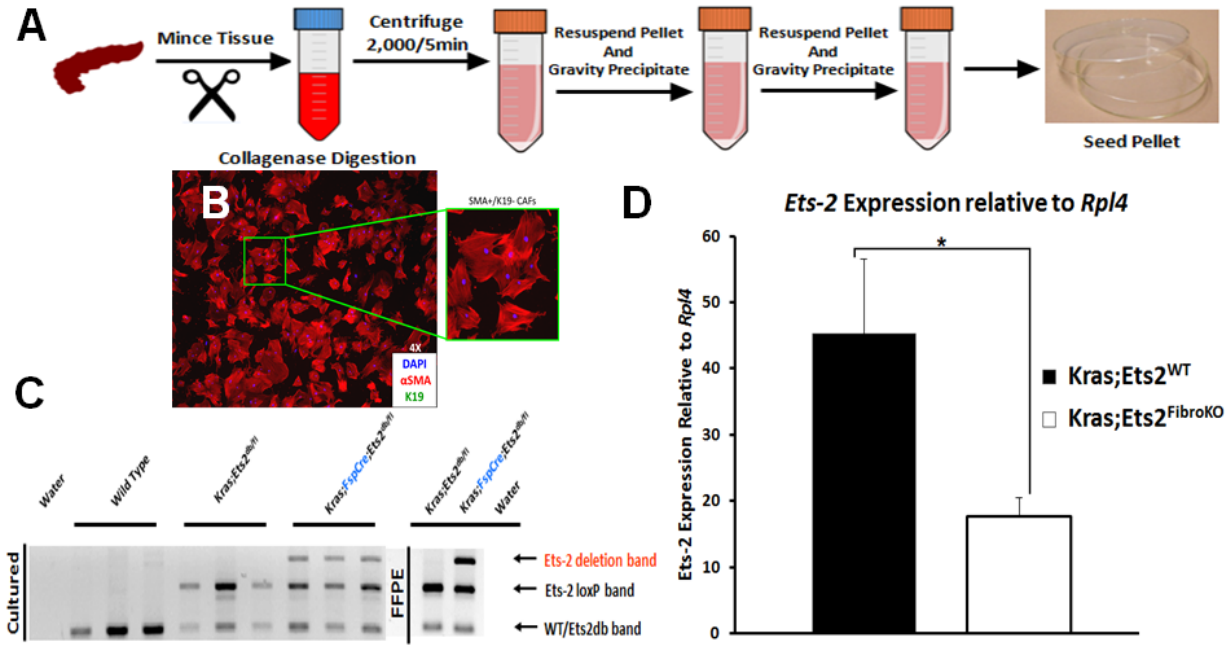

Figure 2. Ets-2^{flox} Allele is Efficiently Deleted in pancreatic CAFs.

(A) Procedure for isolating pancreatic CAFs. (B) Primary cultures stain positive for fibroblast marker SMA (red) and lack epithelial marker K19 (green). (C) Genotyping PCR showing Ets-2 deletion specific band in both cultured pancreatic fibroblasts and formalin fixed parafin embedded (FFPE) tissue. (D) Quantitative Real Time PCR showing *Ets-2* deletion in pancreatic CAFs harvested from Kras;Ets-2^{FibroKO} mice. (* $p<0.05$)

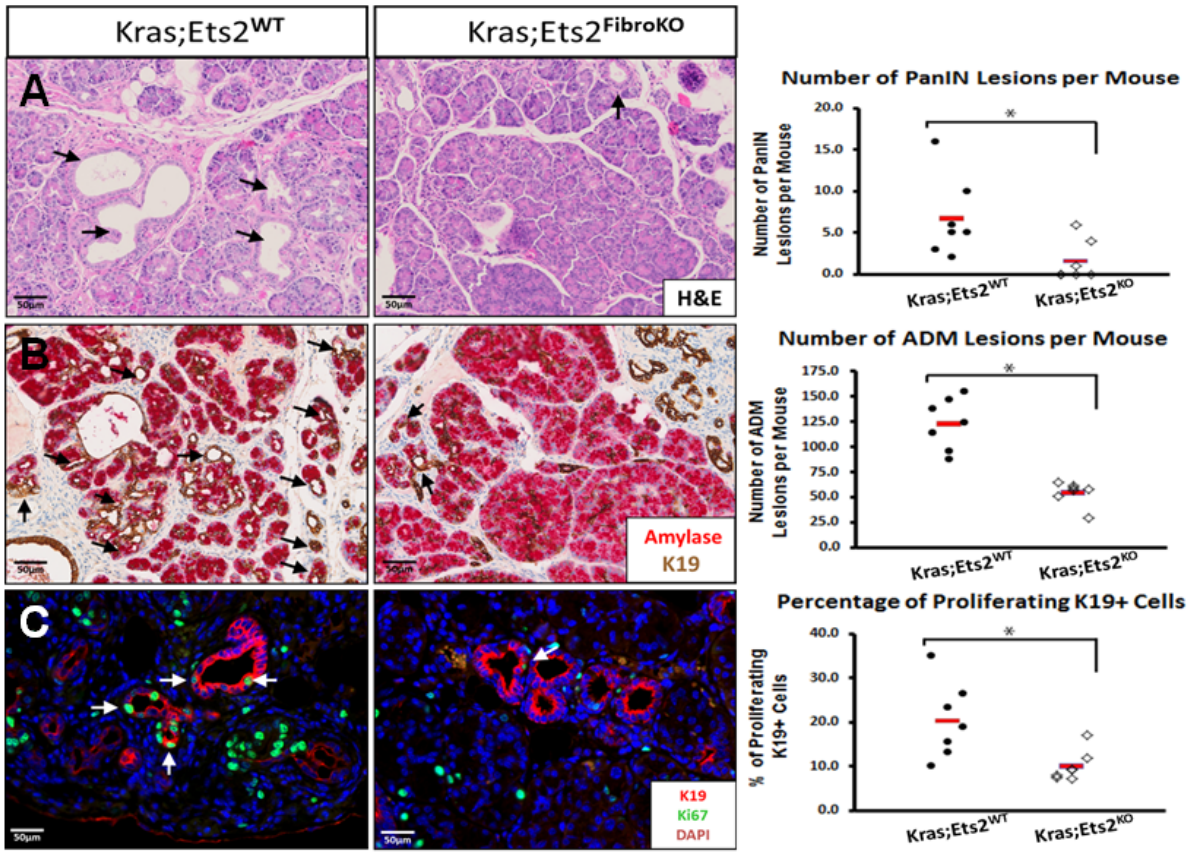

Figure 3. Ets-2^{FibroKO} in Pancreatic CAFs Decreases Premalignant Lesions.

(A) H&E staining indicates that Kras;Ets-2^{FibroKO} mice have decrased PanIN lesions. (B) Dual IHC staining for acini marker amlyase (red) and ductal marker (brown) shows that Kras;Ets-2^{FibroKO} mice have decreased acinar-to-ductal metasplasic (ADM, amylase/K19 double positive) lesions. (C) Dual IF staining for ductal cell marker K19 (red) and proliferation marker Ki67 (green) showing that Kras;Ets-2^{FibroKO} mice have decreased percentage of proliferating ductal cells. (Arrows indicate PanIN (A) ADM (B) or proliferating tumor cells (C) respectively. Red bars depict averages; *p<0.05, n=7)

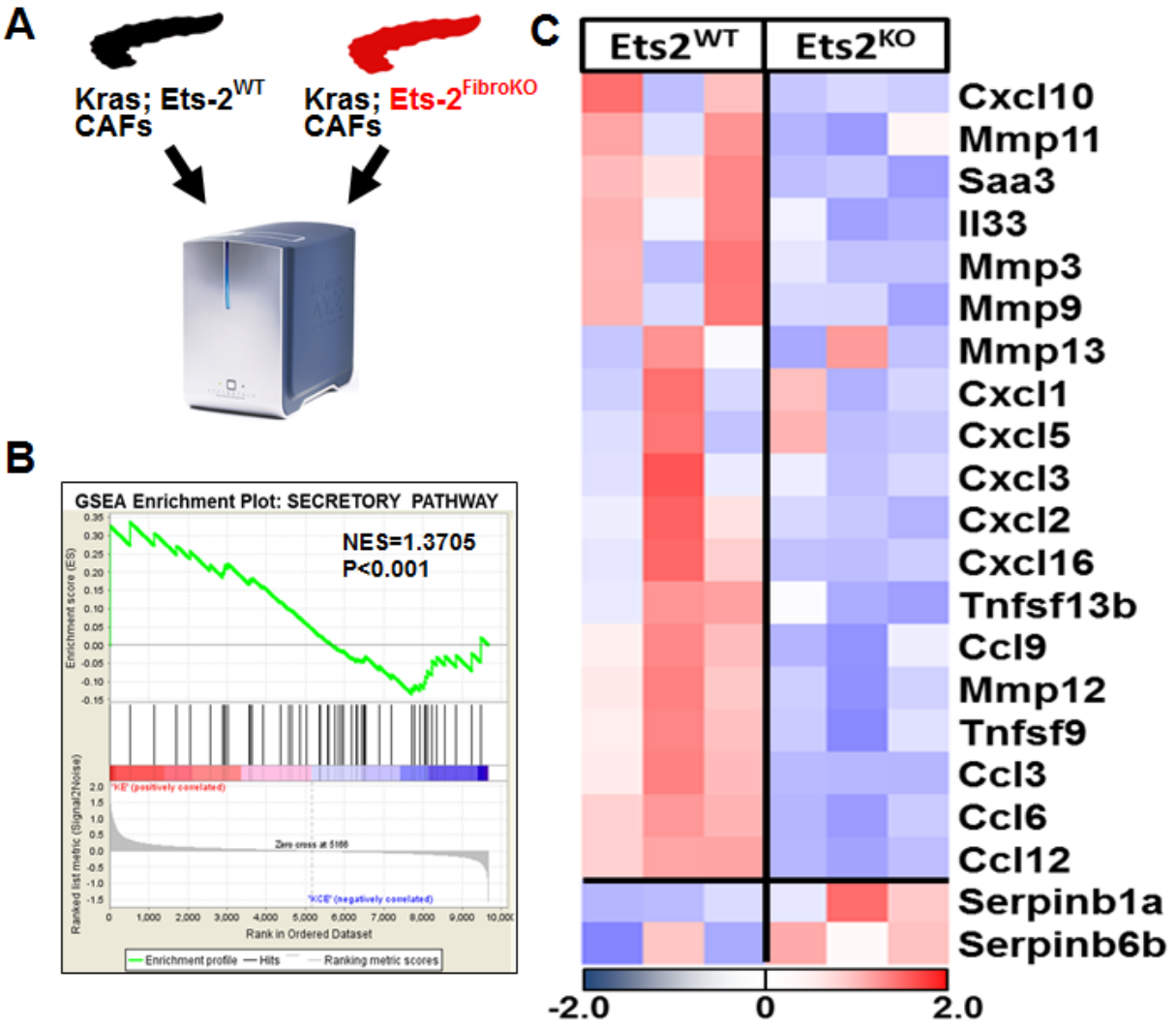

Figure 4. Ets-2 CAF Deletion Decreases Pro-Tumor Secretome.

(A) Cartoon depiction of microarray experiment between Ets^{WT} and Ets^{FibroKO} CAFs[4]. (B) Gene Set Enrichment Analysis (GSEA) plot showing mis-regualtion of secretome in Ets-2^{FibroKO} pancreatic CAFs. (B) Heatmap depicting a decrease in pro-tumor secretome in Ets-2^{FibroKO} CAFs.

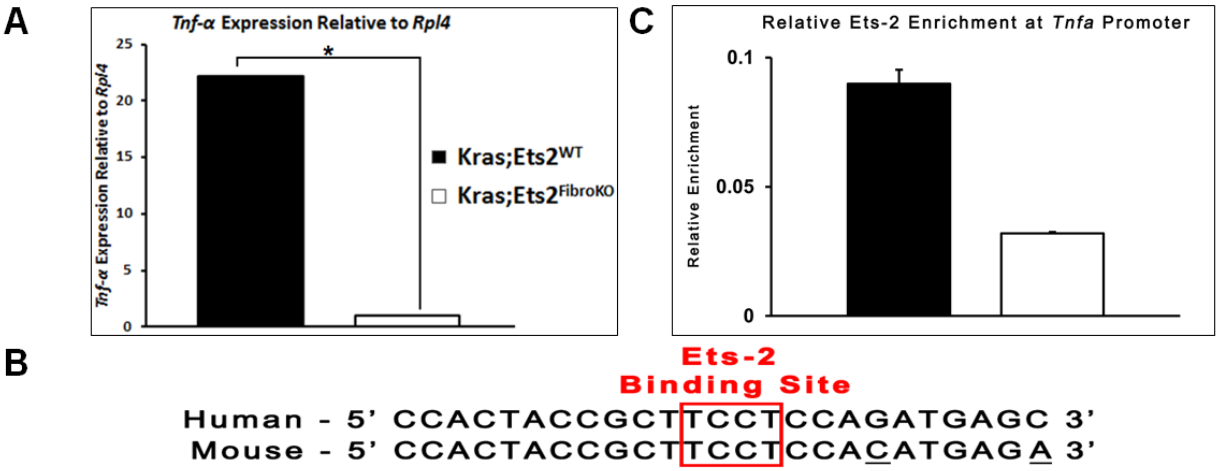

Figure 5. Ets-2 Regulates TNF-α in pancreatic CAFs.

(A) Quantitative Real Time PCR showing *TNF-α* expression is dramatically decreased in Ets-2^{FibroKO} CAFs. (B) *TNF-α* promoter contains a evolutionarily conserved putitive Ets-2 binding site. (C) Chromatin immunoprecipitation (ChIP) against Ets-2 shows 2.8-fold enrichment at the *Tnfa* promoter. (* $p<0.05$)

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